# Crucial role for TNF receptor-associated factor 2 (TRAF2) in regulating NFκB2 signaling that contributes to autoimmunity

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**TNF receptor-associated factor 2 (TRAF2) is a key intracellular signaling mediator that acts downstream of not only TNFα but also various members of the TNFα superfamily. Here, we report that, despite their lack of TNFα signaling, TRAF2$^{-/-}$TNFα$^{-/-}$ mice develop an inflammatory disorder characterized by autoantibody accumulation and organ infiltration by T cells with the phenotypes of activated, effector, and memory cells. RAG1$^{-/-}$ mice reconstituted with TRAF2$^{-/-}$TNFα$^{-/-}$ bone marrow cells showed increased numbers of hyperactive T cells and rapidly developed progressive and eventually lethal inflammation. No inflammation was observed in RAG1$^{-/-}$ mice reconstituted with TRAF2$^{-/-}$TNFα$^{-/-}$T-cell receptor β$^{-/-}$ or TRAF2$^{-/-}$TNFα$^{-/-}$NFκB-induced kinase$^{+/-}$ bone marrow cells. The pathogenic TRAF2$^{-/-}$TNFα$^{-/-}$ T cells showed constitutive NFκB2p52 activation and produced elevated levels of T-helper 1 and T-helper 17 cytokines. Our results suggest that a regulatory circuit consisting of TRAF2–NFκB-induced kinase–NFκB2p52 is essential for the proper control of effector T-cell polarization and that loss of T-cell TRAF2 function induces constitutive NFκB2p52 activity that drives fatal autoimmune inflammation independently of TNFα signaling. The involvement of this regulatory circuit in controlling autoimmune responses highlights the delicate balance required to avoid paradoxical adverse events when implementing new targeted anti-inflammatory therapies.**

TNF receptor-associated factor 2 (TRAF2) is an important adaptor protein and an E3 ubiquitin ligase. Our previous studies of TRAF2-deficient mice showed that TRAF2 is essential for mediating cell survival, normal adaptive immune responses, and lymphocyte homeostasis (1, 2). We previously generated TRAF2$^{-/-}$TNFα$^{-/-}$ double knockout (DKO) mice and demonstrated that the survival of TRAF2-deficient mice is greatly improved by eliminating TNFα signaling (1, 2). Unexpectedly, many TRAF2$^{-/-}$TNFα$^{-/-}$ mice manifest an inflammatory phenotype as they age. We therefore postulated that TRAF2 deficiency may either disrupt or derepress harmful signaling pathways that operate independently of TNFα, such as noncanonical NFκB2(p100/p52) signaling that spontaneously induces chronic inflammation (3).

Previously, Grech et al. demonstrated that B-cell–specific TRAF2 KO mice showed increased B-cell survival concomitant with constitutive NFκB2 activation (4). Another study, by Gardam et al., showed that TRAF2 and TRAF3 coordinate and negatively regulate B-cell–activating factor of the TNF family (BAFF) signaling in B cells, suggesting that TRAF proteins may cooperate to control signaling downstream of TNF receptor superfamily members (5). More recent studies have linked TRAF2 and TRAF3 to the control of NFκB-induced kinase (NIK), which regulates the processing of NFκB2p100 to NFκB2p52 (6, 7). Studies of NIK-deficient mice have also supported the notion that the p100 form of NFκB2 can serve as a negative feedback regulator for the classical NFκB1 signaling pathway triggered during T-cell receptor (TCR)-mediated T-cell activation (8). Interestingly, NIK-deficient mice are prone to autoimmunity because of a defect in the production of regulatory T cells (9). In addition, transgenic mice constitutively expressing NFκB2p52 in lymphocytes develop inflammatory autoimmune disease (3). These results indicate that the threshold of NFκB2 signaling must be properly controlled to keep the immune system in check and that TRAF2 appears to be a key player in this complex regulatory circuit.

In this study, we found that the constitutive NFκB2 activity resulting from TRAF2 ablation led to aberrant T-cell activation and a skewing toward T-helper 1 (Th1)/Th17 effector T-cell polarization that accounted for the autoimmune inflammatory response. Moreover, this phenotype could be reversed by a haplodeficiency of NIK. Our results show that a delicate balance of TRAF2/NIK/NFκB2 signaling controls T-cell inflammatory responses independently of TNFα and that proper manipulation of this balance will be needed to achieve effective new treatments for autoimmune and chronic inflammatory disorders.

## Results

**TRAF2 Deficiency Results in Chronic Inflammation and a Defect in Peripheral T-Cell Tolerance Independent of TNFα Signaling.** TRAF2$^{-/-}$TNFα$^{-/-}$ (DKO) mice were born at less than the expected Mendelian ratio (Table S1), and many of the survivors had abnormally short life spans (Fig. 1*A*). Some of these DKO mice showed the small body size, ruffled fur, and wasted appearance that is often associated with chronic inflammation. Histological examination of these animals at age 6 wk revealed significant lymphocyte infiltration into the lungs and liver (Fig. 1*B* and Fig. S1*A*). At about 6 months of age, several DKO mice manifested distension of the abdominal cavity and eye inflammation (Fig. S1*B*), confirming that the chronic inflammation persists and increases in intensity as the mice age. Serum anti-dsDNA and anti-histone antibodies were increased in DKO mice (Fig. S2). These data suggested that, even in the absence of TNFα signaling, TRAF2 deficiency leads to chronic inflammation and defects in immune tolerance that eventually compromise viability.

Examination of T-cell subsets among the peripheral lymphocytes from DKO mice and littermate controls by flow cytometry (FACS) revealed that the effector/memory (CD44$^{hi}$CD62L$^{lo}$) and activated (CD69$^{+}$) populations among total lymph node

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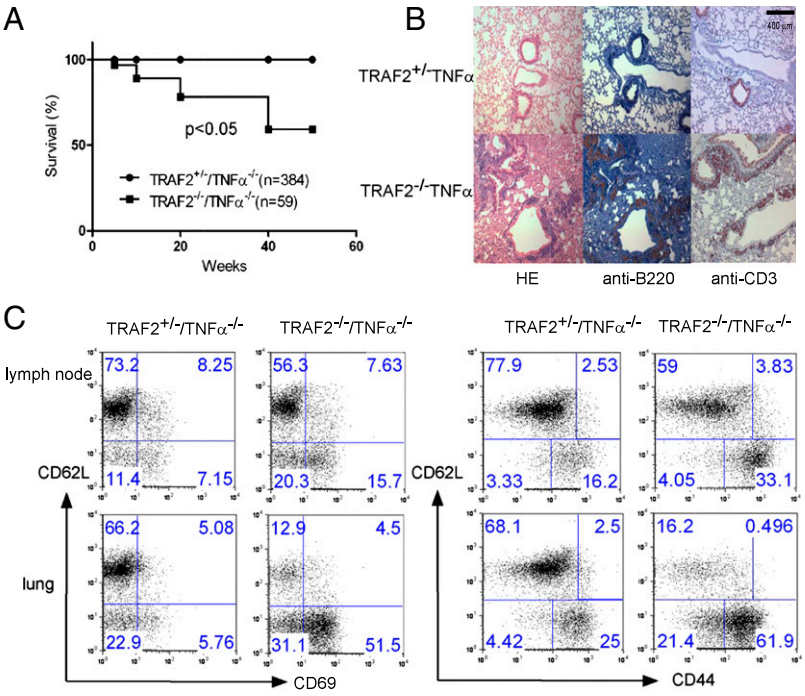


**Fig. 1.** Development of fatal inflammatory disease in TRAF2$^{-/-}$TNFα$^{-/-}$ (DKO) mice. (*A*) Reduced survival. TRAF2$^{-/-}$TNFα$^{-/-}$ (DKO) and TRAF2$^{+/-}$TNFα$^{-/-}$ (littermate control) mice were monitored for survival for over 1 y. Results are expressed as the percentage of original mice remaining viable. (*B*) Lymphocyte infiltration. Lung sections from 4-mo-old DKO and control mice were stained with H&E to show gross structure (*Left*), with anti-B220 to detect B cells (*Center*), and with anti-CD3 to detect T cells (*Right*). (Scale bar applies to all images.) (*C*) Flow cytometric determination of surface expression of CD44, CD62L, and CD69 proteins on T-cell subsets from LN and lungs of DKO and TRAF2$^{+/-}$TNFα$^{-/-}$ (littermate control) mice. Numbers in quadrants indicate the percentage of total CD4$^+$ T cells represented by the subset of interest. For *B* and *C*, data shown are representative of at least five independent experiments.

(LN) and lung DKO CD4$^+$ T cells were markedly increased (Fig. 1*C*). Analysis of serum cytokines showed that DKO mice had elevated levels of IL-1, IL-2, IL-6, IL-12, IL-17, and IFNγ (Fig. S3). Altogether, the combined presence in DKO mice of increased activated-, effector-, and memory-type T cells, elevated serum autoantibodies, and up-regulated Th1 and Th17 cytokines suggests that these mutants suffer from a breakdown in T-cell tolerance. These data thus demonstrate a previously unrecognized role for TRAF2 as an immune tolerance regulator that acts independently of the TNFα pathway to help prevent severe inflammatory disease.

**TRAF2 Function in T Cells Is Required to Prevent Autoimmune Inflammation.** To determine whether the inflammatory phenotype of DKO mice resulted from an intrinsic defect in immune cells originating from hematopoietic stem cells, we generated RAG1$^{-/-}$ chimeric mice that were reconstituted with bone marrow (BM) cells from DKO mice or their control littermates. FACS analysis of peripheral lymphocytes from DKO chimeras revealed significant increases in activated-, effector-, and memory-type CD4$^+$ T cells (Fig. S4*A*). Starting at 5 wk postreconstitution, DKO chimeras developed diarrhea, hunched back, hair loss, and ruffled coat (Fig. S4*B*), a constellation of features consistent with the acute onset of progressive inflammatory disease. The chimeric mutants showed a dramatic loss in weight by age 5 wk (Fig. 2*A*). In contrast, mice reconstituted with control BM cells remained healthy throughout the entire 8-wk observation period. At 4 wk postreconstitution, we isolated the colons of some DKO chimeras and noted marked hemorrhaging and swelling indicative of severe colitis (Fig. S4*C*). Histological analysis of surviving (but sick) DKO chimeras at 6 wk postreconstitution revealed massive mononuclear infiltrates in lung and colon (Fig. 2*B*). By 8 wk postreconstitution, fully 90% of DKO chimeras were dead (Fig. 2*C*). To confirm that the aggressive inflammation in DKO chimeras was caused by an intrinsic T- or B-cell defect, we first established DKO mice in the TCRβ$^{-/-}$ background and examined the phenotypes of RAG1$^{-/-}$ recipients reconstituted with BM cells from DKO/TCRβ$^{+/-}$ or DKO/TCRβ$^{-/-}$ mice. Like DKO chimeras, DKO/TCRβ$^{+/-}$ chimeras (T cells intact) showed hunched back, rough fur, and reduced weight by 5 wk postreconstitution (Fig. S4*D*). In contrast, DKO/TCRβ$^{-/-}$ chimeras, which lack functional T cells, remained healthy throughout the entire observation period. Thus, the lethal inflammatory disease in DKO chimeras truly depends on T cells.

**Dysregulated NFκB2 Signaling and Cytokine Production in DKO T Cells.** To determine whether TRAF2 is a T-cell intrinsic regulator, we first examined the expression pattern of TRAF2 in WT T cells after TCR engagement and found that TRAF2 was strongly induced in a time-dependent fashion by anti-CD3/CD28 antibody cross-linking (Fig. S5*A*). We then investigated the effect of TRAF2 ablation on the canonical NFκB1 and noncanonical NFκB2 signaling cascades in T cells. As expected, IκBα degradation proceeded in the expected time-dependent fashion in stimulated littermate control CD4$^+$ T cells but was significantly impaired in stimulated DKO CD4$^+$ T cells (Fig. 3*A*). In addition, nuclear RelA/p65 levels were dramatically decreased in DKO CD4$^+$ T cells after anti-CD3/CD28 stimulation (Fig. S5*B*). In contrast, components of the noncanonical NFκB2 signaling pathway (p52 and RelB) were increased in DKO CD4$^+$ T cells (Fig. S5*B*). Consistently, DKO CD4$^+$ T cells exhibited increased NFκB2p100 processing even in the absence of exogenous TCR stimulation (Fig. 3*A*). Thus, we postulate that the constitutive processing of NFκB2p100 in DKO CD4$^+$ T cells may be the major driving signal that forces the expansion of activated-, effector-, and memory-like T cells.

To determine the effects of TRAF2 loss on cytokine expression induced by TCR engagement, we examined intracellular cytokine production in isolated DKO and littermate control CD4$^+$ T cells treated with phorbol myristate acetate (PMA)/ionomycin in vitro. At 4 h after PMA/ionomycin stimulation, significant increases in the percentages of IL-17$^+$ (2.42%) and IFNγ$^+$ (1.88%) subsets were observed among stimulated DKO CD4$^+$ T cells (Fig. 3*B*). Interestingly, marked increases in the percentages of IL-17$^+$ and IFNγ$^+$ cells were also observed among unstimulated DKO CD4$^+$ T cells (Fig. 3*B*), an abnormality that likely contributes to the progressive inflammation observed in DKO mice. Similarly, IL-2 mRNA was already greatly increased in DKO CD4$^+$ T cells compared with controls before anti-CD3/CD28 stimulation (Fig. S5*C*). However, isolated DKO CD4$^+$ T cells stimulated with anti-CD3/CD28 showed impaired, rather than enhanced, TCR-induced T-cell pro-

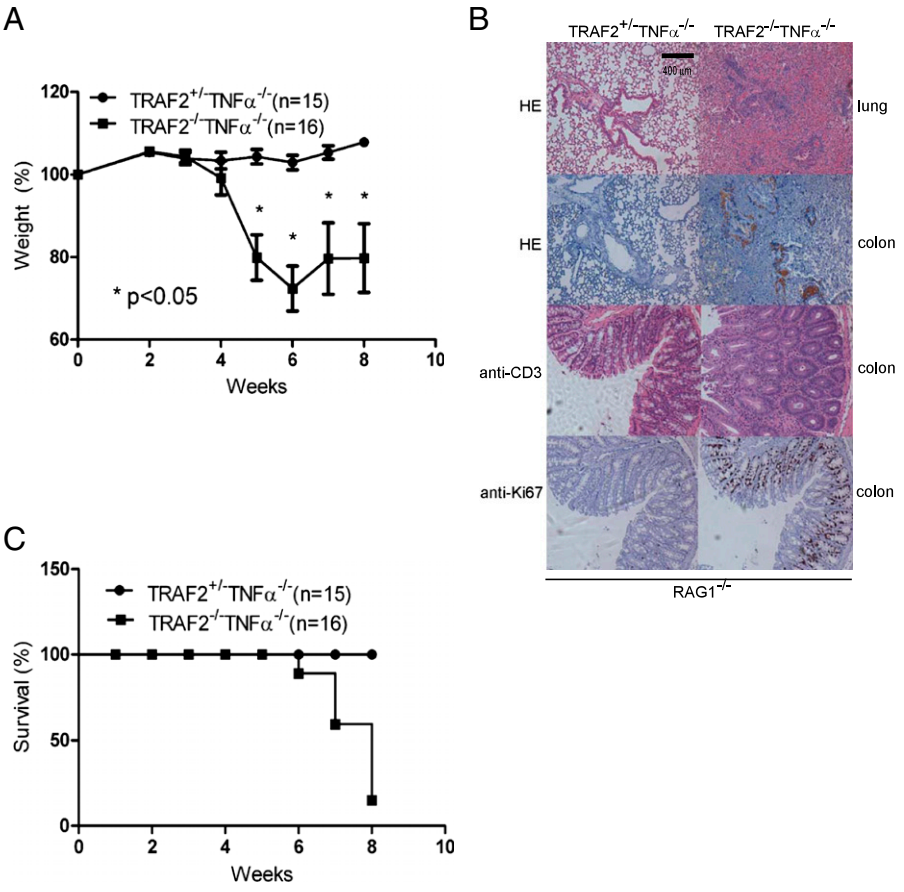


**Fig. 2.** RAG1$^{-/-}$ chimeras reconstituted with TRAF2$^{-/-}$TNFα$^{-/-}$ BM cells recapitulate the phenotypes of DKO mice. (*A*) Decreased body weight. The body weights of the TRAF2$^{-/-}$TNFα$^{-/-}$RAG1$^{-/-}$ chimeric mice (DKO chimeras) and TRAF2$^{+/-}$TNFα$^{-/-}$RAG1$^{-/-}$ littermate control chimeric mice were monitored weekly starting at 1 wk postreconstitution. Data are mean mouse body weight ± SD expressed as a percentage of the original mean weight at week 0. *$P < 0.05$. Results shown are from a single experiment involving 15–16 mice per genotype. (*B*) Lymphocyte infiltration. Lung and colon sections from DKO chimeras and controls ($n = 5$ per group) were examined histologically at 6 wk postreconstitution. Lungs and colon were stained with H&E to show gross structures. Colons were immunostained with anti-CD3 to detect T cells and with anti-Ki67 to detect damage-induced epithelial proliferation. (Scale bars apply to all images.) Data shown are representative of two independent experiments. (*C*) Decreased survival. The survival of the chimeric mice in *A* was monitored weekly starting at 1 wk postreconstitution. Data are percentage survival. *$P < 0.05$.

liferation (Fig. S5*D*). Altogether, these data suggest that the loss of TRAF2 in DKO CD4$^+$ T cells impairs canonical NFκB1 signaling but constitutively activates noncanonical NFκB2 signaling.

Next, we used quantitative real-time PCR (qPCR) to examine the mRNA expression of a broad range of cytokines by DKO and control CD4$^+$ T cells. As expected, there was a significant increase in DKO CD4$^+$ T cells in the basal expression of several cytokine mRNAs, including IFNγ, IL-17, IL-21, and IP-10 (Fig. 3*C*). Upon anti-CD3/CD28 stimulation, levels of IFNγ, IL-17, and IL-21 mRNAs in DKO CD4$^+$ T cells were greatly enhanced compared with stimulated control T cells (Fig. 3*C*). More importantly, this robust induction of IL-21 mRNA in DKO CD4$^+$ T cells, regardless of stimulation, identified these cells as activated inflammatory lymphocytes, consistent with the previous identification of IL-21 as crucial for the development of various inflammatory diseases (10). Thus, our data suggest that the constitutive NFκB2p100/p52 processing in DKO T cells drives the overexpression of inflammatory cytokines such as IL-17, IFNγ, and IL-21 that skew T-cell differentiation toward Th1/Th17 polarization.

**TRAF2 Is Required for Controlling NFκB2-Mediated Regulation of Naïve and Effector T-Cell Functions.** The production of proinflammatory cytokines by DKO T cells suggested that loss of TRAF2 has a profound impact on effector T-cell function. We then analyzed TRAF2 and NFκB2 expression in sorted WT (C57BL/6 TRAF2$^{+/+}$TNFα$^{+/+}$) naïve (CD4$^+$CD62L$^{hi}$) and effector (CD4$^+$CD62L$^{lo}$) T cells. Immunoblotting revealed that TRAF2 protein was comparable in WT naïve and effector T cells but that NFκB2p100, NFκB2p52, and RelB were all increased in WT effector cells compared with WT naïve cells (Fig. S6). These data imply that proper NFκB2 signaling is required for normal T-cell activation and effector-cell differentiation. Interestingly, the RelB and NFκB2p52 proteins were substantially increased in both DKO naïve and effector T cells compared with control T cells (which exhibited the WT pattern) (Fig. 4*A*). This dramatic and aberrant increase in NFκB2 protein in naïve DKO T cells suggested that they may have undergone premature differentiation into effector T cells, consistent with our FACS data. Surprisingly, the Bim and Bcl-XL proteins were also strikingly elevated in these naïve DKO CD4$^+$ T cells (Fig. 4*A*), a relevant finding because Bim was unexpectedly shown to be required for the activation of autoreactive T cells (11). We speculate that the excessive NFκB2 activation and increased levels of Bim and Bcl-XL in unstimulated DKO T cells lead to their premature acquisition of activated (effector) status, promoting the production of Th1 and Th17 cytokines and autoimmune inflammation.

The transcription factor IFN regulatory factor 4 (IRF4) is essential for IL-21 production and Th17 cell differentiation (12, 13). We therefore examined IRF4 expression in DKO and control naïve and effector T cells. Compared with control effector T cells, DKO effector T cells showed much higher levels of IRF4 expression, in parallel with their aberrant increase of NFκB2 (Fig. 4*A*). Furthermore, levels of IL-21, RORγt, and IL-17 mRNAs were significantly increased in both DKO naïve and effector T cells (Fig. 4*B*). The altered expression of NFκB2 in DKO naïve T cells combined with the marked increases in IL-21, RORγt, and IL-17 mRNAs support a scenario in which aberrant activation of NFκB2 signaling caused by loss of TRAF2 triggers the up-regulation of Th17-related genes that drive the premature differentiation of inflammatory (effector) T cells and the development of autoimmune symptoms.

**Inflammatory Disorder in DKO Chimeric Mice Is NFκB2-Dependent.** Previous studies have clearly demonstrated that the genetic deletion of one NIK allele in mice blocks the postnatal death induced by TRAF2 ablation (6, 7). To determine whether TRAF2/NIK/NFκB2 signaling was also important for the T-cell–dependent inflammatory disorder in our DKO mice, we explored

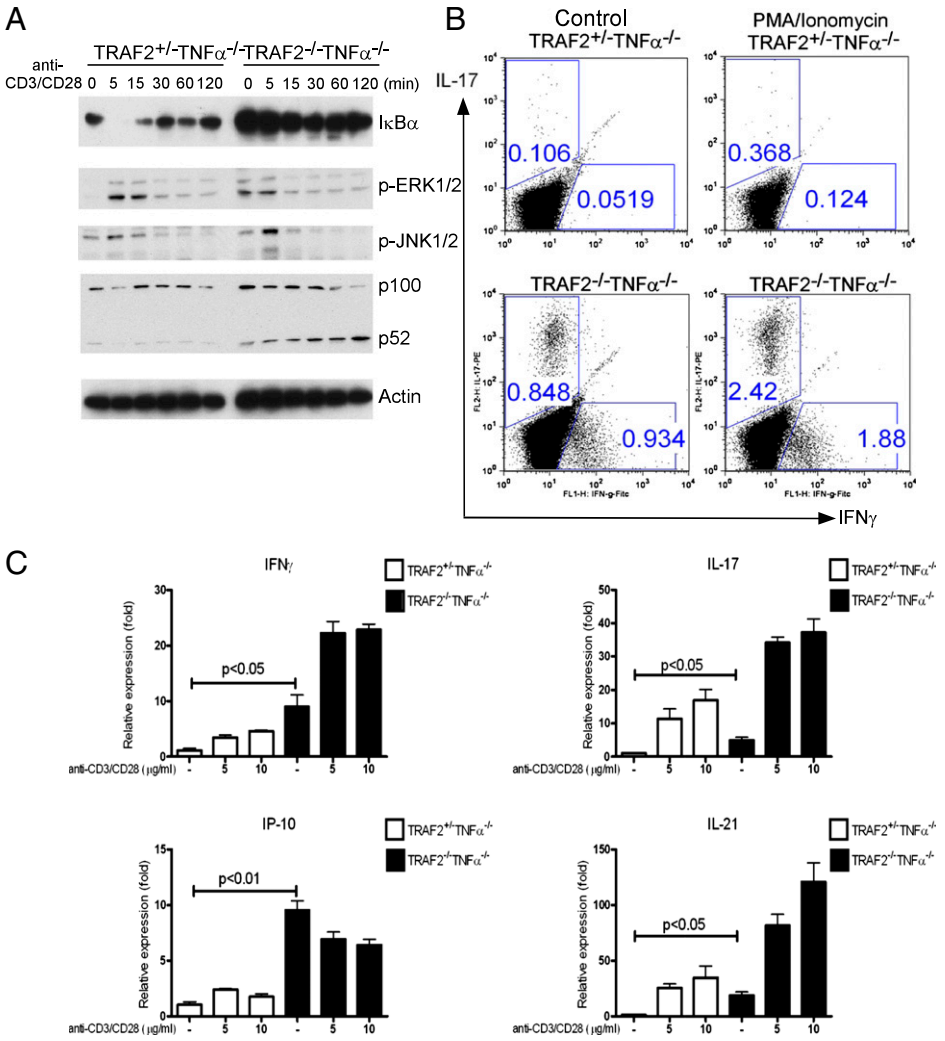


**Fig. 3.** TRAF2 regulates TCR downstream signaling and cytokine expression in T cells. (*A*) Impaired IκBα degradation and increased NFκB2p100 processing. $CD4^+$ T cells were isolated from DKO and control littermate mice and stimulated in vitro with anti-CD3/CD28 for the indicated times. Lysates were immunoblotted to detect expression levels of IκBα, phosphorylated forms of ERK1/2 (p-ERK1/2) and JNK1/2 (p-JNK1/2), NFκB2p100, and NFκB2p52 proteins. Actin was used as loading control. (*B*) Increased Th1 and Th17 cells. DKO and control $CD4^+$ T cells were stimulated in vitro with PMA/ionomycin, and levels of intracellular IFNγ and IL-17 expression were analyzed by flow cytometry. (*C*) Increased inflammatory cytokine mRNA expression. DKO and control $CD4^+$ T cells were stimulated for 12 h with 0, 5, or 10 μg/mL anti-CD3/CD28, and levels of IFNγ, IL-17, IL-21, and IP-10 mRNAs were determined by qPCR. Data shown are the mean ± SD of triplicates and are expressed as fold increase relative to 18S RNA. All results shown are representative of three independent experiments.

the role of NIK in the phenotypes of our DKO mice and DKO/$RAG1^{-/-}$ chimeras. We first attempted to breed $TRAF2^{+/-}$ mice with $NIK^{+/-}$ animals ($Map3k14^{+/-}$ in a pure C57B/6 background), but no viable $TRAF2^{-/-}NIK^{+/-}$ or $TRAF2^{-/-}NIK^{-/-}$ mice were produced over a 2-y observation period. However, we were successful in intercrossing $TRAF2^{+/-}NIK^{+/-}TNFα^{-/-}$ mice to generate $TRAF2^{-/-}NIK^{+/-}TNFα^{-/-}$ (DKO/$NIK^{+/-}$) mice and $TRAF2^{-/-}NIK^{-/-}TNFα^{-/-}$ (TKO) mice. Although DKO/$NIK^{+/-}$ mice remained healthy and did not manifest any developmental abnormalities (Fig. 5*Aa* and Fig. S7), the TKO mutants were sickly from birth and rapidly developed alopecia, hunched back, and psoriasis-like skin inflammation (Fig. 5*Ab* and Fig. S7). Histological analysis revealed heavy infiltration of mononuclear cells into multiple organs of TKO mutants, including skin (Fig. 5*Ad*) and lung (Fig. 5*Bd*). The TKO mice eventually developed autoimmune symptoms and died within 8 wk. In contrast, histological analysis of skin and lung tissues from DKO/$NIK^{+/-}$ mice revealed a normal phenotype with no sign of the major inflammatory infiltrates observed in DKO and TKO mice (Fig. 5 *Ac* and *Bc*). These data suggest that there is a delicate balance of TRAF2/NIK/NFκB2 signaling that sustains T-cell tolerance and that disruption of this balance in any way can lead to the development of lethal autoimmune disease.

Analysis of lymphoid cell populations in DKO/$NIK^{+/-}$ mice showed no differences in $CD4^+CD69^+$ and $CD8^+CD69^+$ T-cell subsets compared with their TRAF2-expressing littermate controls (Fig. S8*A*). Similarly, the ablation of one NIK allele reduced the elevated expression of IFNγ and IL-17 that was observed in DKO $CD4^+$ T cells (Fig. S8*B*). Because NIK controls the processing of NFκB2 (7), our results imply that the normal phenotypes of DKO/$NIK^{+/-}$ mice result from the genetic attenuation of the aberrant NFκB2 activation induced by loss of TRAF2. To further investigate the role of NIK ablation in preventing lethal inflammation, we reconstituted $RAG1^{-/-}$ mice with BM cells from $TRAF2^{+/-}TNFα^{-/-}$, $TRAF2^{-/-}TNFα^{-/-}$ (DKO), $TRAF2^{+/-}NIK^{+/-}TNFα^{-/-}$, or $TRAF2^{-/-}NIK^{+/-}TNFα^{-/-}$ (DKO/$NIK^{+/-}$) mice. Consistent with our previous observations, DKO chimeras quickly exhibited a hunched back, weight loss, alopecia, and diarrhea. In contrast, DKO/$NIK^{+/-}$ chimeras remained healthy and did not develop any inflammatory symptoms during the entire 6-mo observation period (Fig. 5*C*). Thus, the fatal inflammation induced by loss of TRAF2 is indeed attributable to dysregulation of the NFκB2 signaling pathway. Our observations expose a vital function of TRAF2 in balancing NFκB2 signaling in T cells for preventing fatal autoimmune inflammation.

## Discussion

We previously demonstrated that TNFα deficiency significantly increases the survival rate of TRAF2 KO mice, although it is unable to prevent the development of chronic inflammation in these mutants. Our current work with reconstituted DKO chimeras has shown that the rapid onset of lethal inflammatory disease is intrinsic to hematopoietic cells. Our crosses to $TCRβ^{-/-}$ mice have established that elimination of DKO T cells prevents this inflammation, directly linking DKO T cells to the autoimmune phenotype. These results therefore clearly distinguish be-

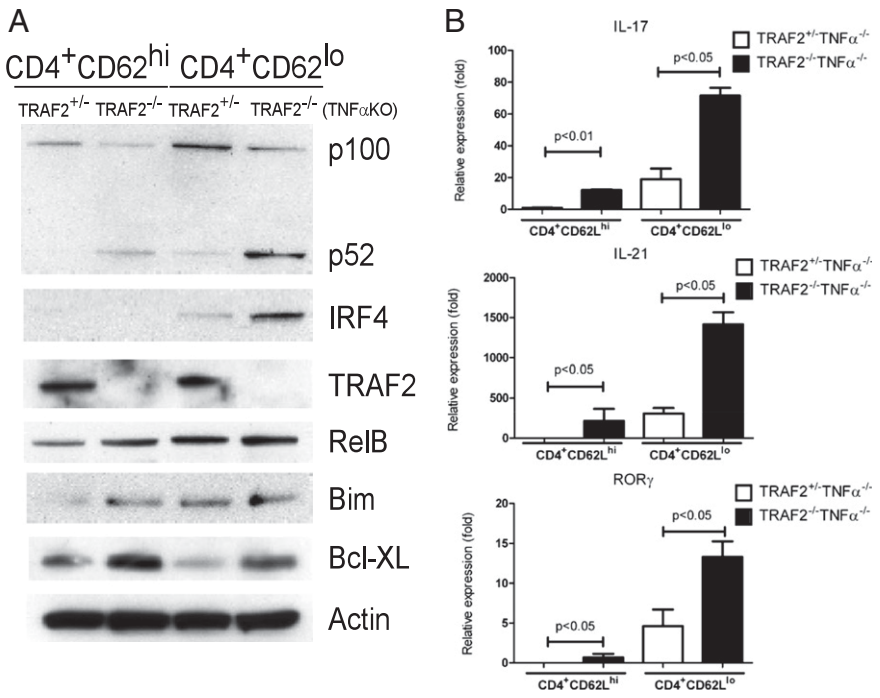


**Fig. 4.** Molecular impact of TRAF2 deficiency on $CD4^+CD62L^{hi}$ and $CD4^+CD62L^{lo}$ T cells. (*A*) Increased IRF4 and NFκB2 in DKO T cells. DKO and $TRAF2^{+/-}TNF\alpha^{-/-}$ (littermate control) $CD4^+CD62L^{hi}$ and $CD4^+CD62L^{lo}$ T cells were immunoblotted to detect the indicated proteins. (*B*) TRAF2 deficiency augments Th17-related gene expression. DKO and littermate control $CD4^+CD62L^{hi}$ and $CD4^+CD62L^{lo}$ T cells were subjected to qPCR to detect expression levels of IL-17, IL-21, and RORγ mRNAs as described in Fig. 3*C*. All results shown are representative of two independent experiments.

tween the functions of TRAF2 and its related family member TRAF3 in suppressing autoimmune inflammatory responses and show that distinct immune cell types are involved (14). For example, a previous study demonstrated that TRAF2 KO mice showed an expansion of marginal-zone B cells but decreased splenic B cells (6, 7). However, this study did not comment on whether these animals showed any signs of inflammatory disease. Interestingly, a study of B-cell–specific TRAF3 KO mice showed that the mutants exhibited splenomegaly and expanded marginal-zone B cells with increased NFκB2 activity (14). As they aged, these B-cell–specific TRAF3 KO mice developed an autoimmune disease characterized by increased anti-dsDNA antibodies and immune complex deposition in the kidney. Thus, activated TRAF3-deficient B cells alone can initiate the development of an autoimmune disease that is most likely driven by the constitutive NFκB2 activation. Interestingly, the early death induced by loss of either TRAF2 or TRAF3 can be prevented by deleting one NIK allele (6, 7), implying that constitutive activation of the noncanonical NFκB2 pathway is the main driver of the postnatal deaths of TRAF2 KO and TRAF3 KO mice. Also, the inflammatory phenotypes of DKO mice and DKO chimeras can be rescued by ablating one NIK allele. Thus, our results are consistent with previous reports and firmly establish that TRAF2 is an essential and direct negative regulator of the NFκB2 pathway. At the cellular level, we found that TRAF2 is up-regulated in WT $CD4^+$ T cells in response to TCR engagement (Fig. S5*A*), suggesting that TRAF2 has a T-cell–intrinsic role in the maintenance of immune homeostasis. Numbers of memory- and effector-type cells were increased among DKO $CD4^+$ T cells (Fig. 1*C*), correlating with the enhanced NFκB2p52 processing observed in total DKO $CD4^+$ T cells (Fig. 3*A*). Thus, the dysregulated NFκB2 signaling in DKO mice may push T cells over the limit of peripheral tolerance control and result in chronic inflammation.

Our results also showed that NFκB1 signaling downstream of TCR engagement was significantly impaired in DKO $CD4^+$ T cells (Fig. 3*A* and Fig. S5*B*). This contrasting effect of TRAF2 deficiency on the canonical and noncanonical NFκB pathways leads us to propose that the NFκB2 pathway is abnormally dominant over NFκB1 signaling in DKO $CD4^+$ T cells and that a crucial function of TRAF2 in WT T cells is to direct signals initiated by TCR engagement first into the NFκB1 pathway and then into the NFκB2 pathway. This hypothesis is supported by a previous report demonstrating the sequential involvement of NFκB1 and NFκB2 in the regulation of TCR activation (8). Our results

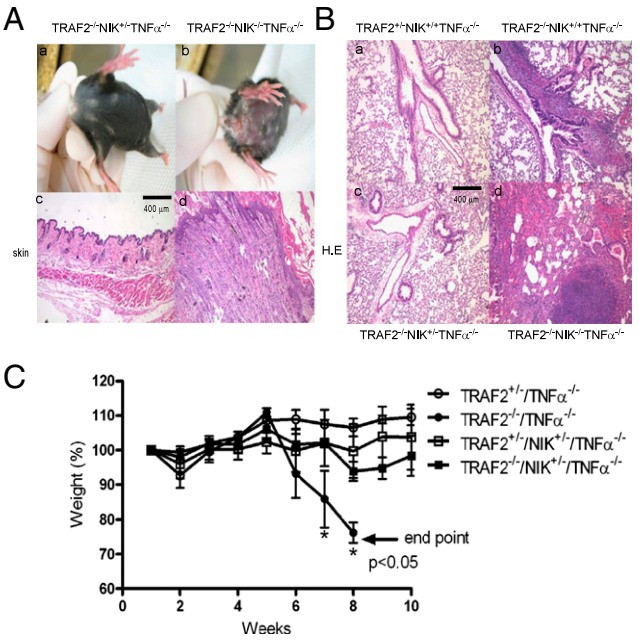


**Fig. 5.** Deletion of one NIK allele ameliorates inflammatory disease in DKO mice and DKO chimeras. (*A*) Comparison of DKO and TKO mice. (*Upper*) Gross appearance. (*Lower*) H&E staining of mouse skin. TKO mice (*b* and *d*) exhibited psoriasis-like inflammation in the skin that was absent from $DKO/NIK^{+/-}$ mice (*a* and *c*). (*B*) Lymphocyte infiltration. Lung sections from $TRAF2^{+/-}NIK^{+/+}TNF\alpha^{-/-}$ (*a*), $TRAF2^{-/-}NIK^{+/+}TNF\alpha^{-/-}$ (*b*), $TRAF2^{-/-}NIK^{+/-}TNF\alpha^{-/-}$ (DKO/$NIK^{+/-}$) (*c*), and $TRAF2^{-/-}NIK^{-/-}TNF\alpha^{-/-}$ (TKO) (*d*) mice were stained with H&E and examined for the presence of lymphocytes. (Scale bar applies to all images.) For *A* and *B*, results shown are representative of two independent experiments involving three mice per group. (*C*) $RAG1^{-/-}$ mice were reconstituted with BM cells from mice of the indicated genotypes ($n$ = 5 per group). Mouse body weights were monitored weekly for 8 wk after reconstitution. $DKO/NIK^{+/-}$ chimeras showed no weight loss or inflammation.

also identify TRAF2 as a unique "gatekeeper" of NFκB signaling downstream of TCR activation. For example, both TRAF6 and TRAF2 are major mediators of the TCR/IκB kinase/NFκB1 pathway (15), and deficiency for TRAF6 or TRAF2 (Fig. 3*A*) seriously impairs TCR-induced IκBα degradation (15). However, unlike TRAF2, TRAF6 deficiency has not been directly linked to NFκB2 signaling (16). Similarly, TRAF3 deficiency has no effect on the NFκB1 pathway (17). Thus, TRAF2 appears to have a distinct and vital position in regulating T-cell homeostasis via a unique mechanism of control over NFκB1/2 signaling.

Interestingly, a particularly robust increase in IL-17, RORγt, and IL-21 expression was observed in DKO $CD4^+CD62L^{hi}$ T cells (Fig. 4*B*), indicating that constitutive activation of NFκB2 in naïve DKO T cells was sufficient to induce Th17 differentiation. Furthermore, IL-21 is known to activate Th17-related gene expression in regulatory T cells, thereby eliminating the suppressive function of these cells (18). We believe that the augmented IL-21 induction in our DKO $CD4^+$ T cells may have promoted Th17 differentiation while suppressing regulatory T-cell function, thus driving inflammatory disease development. Similarly, the expression of IRF4, which is essential for IL-17 and IL-21 production, was markedly up-regulated in DKO effector T cells (Fig. 4*A*). Our data in Fig. 4*A* confirm previous work showing that the differentiation of naïve WT $CD4^+$ T cells into effectors requires increased NFκB2p52 activity (8) and that the induction of IRF4 mRNA in *nfkb1*$^{-/-}$ lymphocytes stimulated with anti-CD3/CD28 is normal (19). Because our results show that IRF4 up-regulation in DKO effector T cells relies on the NFκB2 pathway, they provide evidence of a previously unappreciated link between NFκB2 signaling and Th17 differentiation.

We have established that the abnormal Th1/Th17 polarization of DKO effector T cells depends on NFκB2 because the ablation of one NIK allele in our DKO mice successfully prevented the development of the autoimmune phenotype (Fig. 5*C* and Fig. S7). Both the elevated expression of IFNγ and IL-17 and aberrant inflammation were rescued in DKO/$NIK^{+/-}$ mice and reconstituted DKO/$NIK^{+/-}RAG1^{-/-}$ chimeras (Fig. S8*B*). Thus, fine-tuned control of NFκB2 activation is vital not only for embryogenesis but also for effector T-cell differentiation and the maintenance of immune tolerance. Thus, we believe that TRAF2 has a unique and critical role in preventing inflammatory disease development because it blocks unnecessary NFκB2 activation in T cells and thereby heads off deleterious expansion of Th1 and/or Th17 effector cells.

In summary, our DKO mice demonstrate that negative regulation of the NIK/NFκB2 signaling pathway by TRAF2 is important for maintaining immune homeostasis. Despite TNFα ablation, excessive NFκB2 activation in DKO mice enhanced T-cell activation and triggered an autoimmune-like disease characterized by increased Th1 and Th17 cells. Furthermore, the inflammatory phenotype of DKO mice was rescued by deletion of one NIK allele. Thus, TRAF2-mediated control of NIK/NFκB2 signaling is important for both embryogenesis and preventing harmful inflammation. The role identified for TRAF2 by our findings may inspire the development of important new strategies for treating or blocking autoimmune diseases.

## Materials and Methods

**Generation of DKO Mice.** Homozygous $TRAF2^{-/-}TNF\alpha^{-/-}$ DKO mice were generated by crossing $TRAF2^{+/-}$ and $TNF\alpha^{+/-}$ mice as previously described (2). $NIK^{+/-}$ mice were a kind gift from Amgen Inc. and were backcrossed to C57BL/6 mice for six generations. $TCR\beta^{-/-}$ and $RAG1^{-/-}$ mice were obtained from The Jackson Laboratory. All mice were housed in the University Health Network Mouse Facility under specific pathogen-free conditions. All mouse procedures were approved by the University Health Network Institutional Animal Care and Use Committee.

**BM Chimeras.** BM cells were isolated from DKO, DKO/$NIK^{+/-}$, or DKO/$TCR\beta^{-/-}$ mice or their corresponding littermate controls. See *SI Materials and Methods* for details.

**Histology, Serum Cytokines, and Autoantibodies.** Mouse blood and organs were harvested for determination of cytokines and autoantibodies by ELISA and histological examinations. See *SI Materials and Methods* for details.

**Flow Cytometry and Intracellular Cytokines.** For surface marker or cytokine determinations, spleen and LN cells ($1 \times 10^6$) were stained with antibodies (BD Pharmingen) and subjected to flow cytometric analysis. See *SI Materials and Methods* for details.

**T-Cell Isolation and in Vitro Stimulation.** Total $CD4^+$, naïve $CD4^+CD62L^{hi}$, and effector $CD4^+CD62L^{lo}$ T cells were isolated and stimulated as described in *SI Materials and Methods*.

**Immunoblotting and qPCR.** Immunoblotting and qPCR were conducted as described in *SI Materials and Methods*.

**Statistical Analyses.** The Student's *t* test was used for most comparisons of DKO and littermate control cells. The Wilcoxon rank-sum test was used to compare levels of serum cytokines and autoantibodies. The log-rank test was used for the statistical analysis of mouse survival. Values are expressed as the mean ± SD, and $P < 0.05$ was considered statistically significant. Statistical analyses and graphing were performed with GraphPad Prism software.

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